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Geographical Differentiation in Populations of Japanese Dace *Tribolodon hakonensis* Deduced from Allozymic Variation

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ABSTRACT—Allozymic variation of Japanese dace *Tribolodon hakonensis* was examined at 20 loci in 17 populations derived from Hokkaido to Kyushu. Intrapopulational variability indicated by the proportion of loci polymorphic and heterozygosity was close to the standard levels in vertebrates. On the other hand, allelic frequencies at five loci of the twenty were remarkably different among populations, and even replacement of predominant alleles was observed. The extent of interpopulational differentiation was estimated by coefficient of gene differentiation (G_{ST}) and genetic distance (D). Levels of these indices were remarkably high compared with the levels known among populations in many vertebrates. Based on a dendrogram, we classified the populations into three local groups; 1) Northern Group, 2) Southern Group, and 3) Lake Biwa Group. These results suggest that the populations of different local groups have been highly differentiated from each other and the differentiation is due to geographical isolation.

INTRODUCTION

The genus *Tribolodon* which belongs to the family Cyprinidae has a unique characteristic on distribution: it is widely distributed from the upper reaches of rivers to the coastal regions of the sea in Japan and adjacent countries. The other genera of Cyprinidae do not show such a wide distribution. Therefore fishes of *Tribolodon* have been extensively studied from the viewpoints of physiology [1] and ecology [2]. However, their taxonomy had been confused and greatly different among taxonomists [3–5], because the species of this genus have not been morphologically differentiated enough from each other. Our previous study using allozyme markers clearly showed that the four species of *Tribolodon* classified by Nakamura [4] had been genetically differentiated enough as highly as the interspecific levels in vertebrates [6, 7]. Thus, biochemical analysis provided good information as for taxonomy. In addition, we examined *T. hakonensis* from the waters in Fukushima Pref. and Kochi Pref. in the previous work, and found the possibility that this species contained the highly differentiated populations [7].

In this work, we further analysed *T. hakonensis* collected at more locations from Hokkaido to Kyushu, evaluated the level of intraspecific variability and the extent of interpopulational differentiation, and discussed the evolutionary aspects in populations of *T. hakonensis*.

MATERIALS AND METHODS

Specimens

We collected a total of 883 specimens of *T.*

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hakonensis in 17 waters from Hokkaido to Kyushu. Sample locations are shown in Figure 1, and sample sizes are given in Table 3. Fish were caught at a single location within a few days by a gill net, a casting net, and angling, and were frozen by dry ice at once and stored under −20°C. In the Otsuchi, the Monobe, and the Shimanto Rivers, specimens were collected at a few locations, the upper reaches, and the lower reaches or the coastal regions of the sea, which were isolated by dums at present.

Electrophoretic analyses

Horizontal starch gel electrophoresis was performed upon drip from tissue samples as described by Numachi [8]. The following four buffer systems (Table 1) were used: CAPM (citric acid, 4-(3-aminopropyl)-morpholine), pH 6.0 described by Clayton and Tretiak [9]; CAEA (citric acid, N-(3-aminopropyl)-diethanol-amine), pH 7.0 and CT (citric acid, tris), pH 8.0 described by Numachi *et al.* [10]; and TBE (tris, boric acid, EDTA), pH 8.7 described by Numachi [11]. The staining methods were according to Shaw and Prasad [12], and Taniguchi and Numachi [13]. The gels stained were washed and dried by the method of Numachi [14]. A list of proteins analysed, their abbreviations, E. C. numbers, locus designations, tissues, and buffers employed are shown in Table 1. Tissues of skeletal muscle, liver, heart, and blood were used. Mitochondrial and supernatant isozymes of AAT, IDH, MDH, and ME were discriminated.

Locus and allele designation followed Hanzawa *et al.* [15]. The most common alleles in the populations of the Northern Group were designated as *100* or *−100,* and the other alleles were designated by the relative differences in elec-

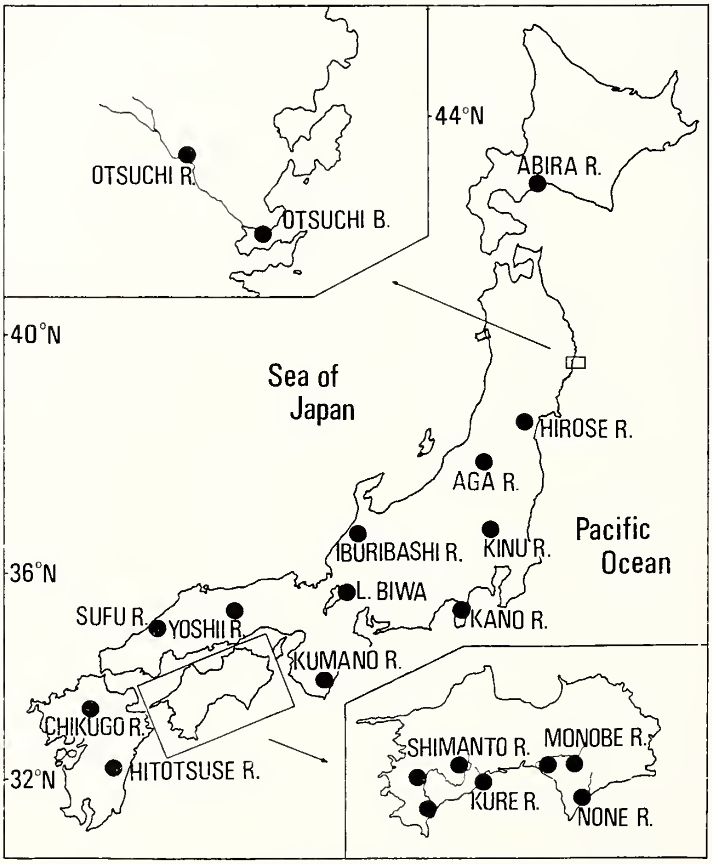

Fig. 1. The sites where specimens of *T. hakonensis* were sampled.

TABLE 1. Enzymatic and non-enzymatic proteins examined, locus designations, tissues, and electrophoretic buffers employed

Protein (Abbreviation; E.C. Number)	Locus	Tissue	Buffer
Aspartate aminotransferase (AAT; 2.6.1.1)	*m-Aat*	muscle	CT
Alcohol dehydrogenase (ADH; 1.1.1.1)	*Adh*	liver	CAEA
α-Glycerophosphate dehydrogenase (α-GDH; 1.1.1.8)	*α-Gdh-2*	liver	CAEA
Glucosephosphate isomerase (GPI; 5.3.1.9)	*Gpi-1*	heart	TBE
	Gpi-2	heart	TBE
Hemoglobin (HB)	*Hb-1*	blood	CT
	Hb-2	blood	CT
Isocitrate dehydrogenase (IDH; 1.1.1.42)	*s-Icd-1*	liver	CAEA
	s-Icd-2	liver	CAEA
Lactate dehydrogenase (LDH; 1.1.1.27)	*Ldh-1*	heart	CT
	Ldh-2	muscle	CT
	Ldh-3	liver	CT
Malate dehydrogenase (MDH; 1.1.1.37)	*s-Mdh-1*	muscle	CAPM
	s-Mdh-2	liver	CAPM
	m-Mdh	muscle	CAEA
Malic enzyme (ME; 1.1.1.40)	*s-Me*	muscle	CAEA
Phosphoglucomutase (PGM; 2.7.5.1)	*Pgm*	muscle	CAEA
6-Phosphogluconate dehydrogenase (6-PGD; 1.1.1.44)	*6-Pgd*	liver	CAEA
Sarcoplasmic protein (SP)	*Sp-2*	muscle	CAEA
	Sp-3	muscle	CAEA

trophoretic mobility of respective gene products with the reference allele, *100* or *−100*.

Statistical analyses

Amount of intrapopulational variability was evaluated by the proportion of loci polymorphic and heterozygosity. Degree of interpopulational differentiation was evaluated by coefficient of gene differentiation (G_{ST}) [16] and genetic distance (D) [17]. G_{ST} value, which is calculated based on average heterozygosity, indicates the degree of allelic differentiation over all populations. D value, which is calculated based on allelic frequencies, indicates the degree of differentiation between pairs of populations. Divergence time between the populations was estimated based on D value and gene substitution rate of 10^{-7} [16].

RESULTS

Intrapopulational variation

Twenty loci controlling 12 kinds of enzymatic and nonenzymatic proteins were examined. The genetic control by 20 loci was mainly deduced from the phenotypes of different species and their hybrids [6, 7, 15]. Allozymic variation was found at 14 of the 20 loci in sample populations of *T. hakonensis*. Particularly, various polymorphisms were observed at 5 loci, *Adh, α-Gdh-2, Gpi-2, s-Mdh-2, 6-Pgd,* and each allelic control was interpreted (Fig. 2). Deviations of observed number of phenotypes from their expectations under Hardy-Weinberg equilibrium were not significant in all sample populations except the upper reach of the Shimanto River (at *6-Pgd*, $P<0.05$).

Intrapopulational variability of *T. hakonensis* indicated by proportion of loci polymorphic and heterozygosity was given in Table 2. The proportion of loci polymorphic ranged from 0.05 to 0.50, and the mean value indicated 0.22±0.10. Heterozygosity observed ranged from 0.019 to 0.085, and the mean value indicated 0.041±0.019. Levels of these two indices were similar among sample populations. Heterozygosity observed agrees with its expectation ($H^o/H^E \fallingdotseq 1$).

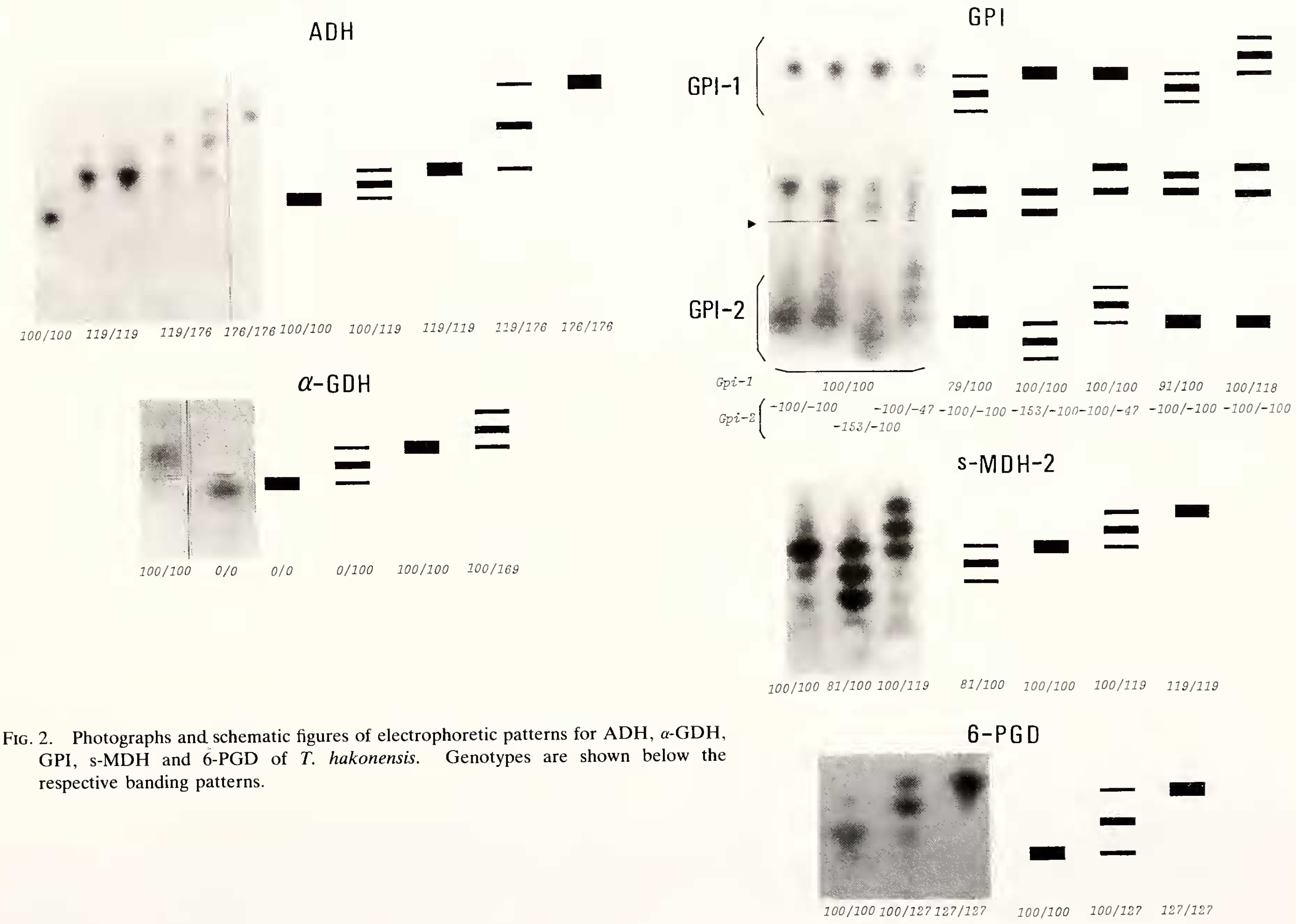

FIG. 2. Photographs and schematic figures of electrophoretic patterns for ADH, α-GDH, GPI, s-MDH and 6-PGD of *T. hakonensis*. Genotypes are shown below the respective banding patterns.

TABLE 2. Estimates of genetic variability at 20 loci within populations of *T. hakonensis*

Location	Proportion of loci polymorphic	Heterozygosity (Observed; H^O)	Heterozygosity (Expected; H^E)	H^O/H^E
Abira R.	0.30	0.039	0.040	0.976
Otsuchi				
upper reach	0.20	0.019	0.020	0.950
Otsuchi Bay	0.30	0.028	0.028	1.000
Hirose R.	0.25	0.046	0.047	0.979
Kinu R.	0.50	0.069	0.075	0.922
Kano R.	0.30	0.072	0.081	0.889
Aga R.	0.25	0.035	0.037	0.952
Iburibashi R.	0.15	0.030	0.029	1.039
Yoshii R.	0.10	0.019	0.019	0.947
Sufu R.	0.20	0.023	0.021	1.098
Chikugo R.	0.15	0.033	0.033	1.014
L. Biwa	0.40	0.085	0.089	0.954
Kumano R.	0.25	0.072	0.074	0.970
None R.	0.15	0.034	0.030	1.125
Monobe R.				
upper reach	0.05	0.026	0.024	1.111
lower reach	0.15	0.029	0.024	1.212
Kure R.	0.10	0.028	0.031	0.917
Shimanto R.				
upper reach	0.25	0.050	0.059	0.848
middle reach	0.20	0.048	0.049	0.974
lower reach	0.20	0.042	0.042	1.000
Hitotsuse R.	0.20	0.034	0.034	1.003
	(0.22±0.10)	(0.041±0.019)		

Interpopulational differentiation

Gene constitution of sample populations collected at 21 locations in 17 waters from Hokkaido to Kyushu were compared (Table 3). Allelic frequencies among subpopulations within the same waters were very similar, and a significant deviation among allelic frequencies was not observed at all loci except *Adh* and *α-Gdh-2* in the Shimanto River population. These subpopulations were isolated by dums at present, and fish of the Otsuchi Bay was particularly the amphidromous type. However, gene constitution of these subpopulations within the same waters was thus very similar.

On the other hand, remarkable differences among populations derived from the different waters were found at five loci, *Adh,α-Gdh-2, Gpi-2, s-Mdh-2, 6-Pgd*. At *Adh* and *α-Gdh-2* of the five, predominant alleles of *100* in the populations of the Pacific coast of the northern Japan and of the coast of the Sea of Japan were replaced with the other alleles (*119* or *0*) in those of the Pacific coast of the southern Japan. Similarly, predominant allele of *100* at *6-Pgd* was replaced with *127* only in the Lake Biwa and the Kumano River populations. Population specific alleles with high frequencies were found, such as *176* at *Adh* in the Lake Biwa population and *−153* at *Gpi-2* in the Kumano River population.

The extent of interpopulational differentiation was evaluated by two kinds of indices, coefficient of gene differentiation (G_{ST}) and genetic distance (D). As for the Otsuchi, the Monobe, and the Shimanto River systems, specimens collected at

Table 3. Allelic frequencies for 14 loci in populations of *T. hakonensis*

Location	*m-Aat*		*Adh*			*α-Gdh-2*			*Gpi-1*				*Gpi-2*			*s-Idh-1*		*s-Idh-2*		
	−100	*−62*	*100*	*119*	*176*	*0*	*100*	*169*	*79*	*91*	*100*	*118*	*−153*	*−100*	*−47*	*52*	*100*	*85*	*100*	*155*
Abira R.	0.988	0.013	0.726	0.274	0.000	0.000	0.977	0.023	0.000	0.000	1.000	0.000	0.000	0.941	0.059	0.023	0.977	0.000	1.000	0.000
Otsuchi																				
upper reach	0.938	0.063	1.000	0.000	0.000	0.000	1.000	0.000	0.000	0.050	0.950	0.000	0.000	0.913	0.088	0.000	1.000	0.000	1.000	0.000
Otsuchi Ray	0.925	0.075	1.000	0.000	0.000	0.000	0.988	0.013	0.000	0.063	0.938	0.000	0.000	0.875	0.125	0.000	1.000	0.013	0.988	0.000
Hirose R.	0.768	0.232	0.938	0.063	0.000	0.000	1.000	0.000	0.012	0.000	0.988	0.000	0.000	0.720	0.280	0.000	1.000	0.000	1.000	0.000
Kinu R.	0.670	0.330	0.826	0.174	0.000	0.080	0.920	0.000	0.030	0.000	0.970	0.000	0.013	0.744	0.244	0.000	1.000	0.000	1.000	0.000
Kano R.	0.829	0.171	0.600	0.400	0.000	0.000	1.000	0.000	0.000	0.000	1.000	0.000	0.000	0.925	0.075	0.000	1.000	0.000	0.975	0.025
Aga R.	1.000	0.000	1.000	0.000	0.000	0.015	0.985	0.000	0.015	0.000	0.985	0.000	0.000	0.712	0.288	0.000	1.000	0.000	1.000	0.000
Iburibashi R.	1.000	0.000	1.000	0.000	0.000	0.288	0.713	0.000	0.000	0.000	1.000	0.000	0.000	0.446	0.554	0.000	1.000	0.000	0.961	0.039
Yoshii R.	1.000	0.000	0.962	0.038	0.000	0.207	0.793	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0.000
Sufu R.	1.000	0.000	0.864	0.136	0.000	0.093	0.907	0.000	0.000	0.000	1.000	0.000	0.000	0.988	0.012	0.000	1.000	0.000	1.000	0.000
Chikugo R.	1.000	0.000	0.591	0.409	0.000	0.239	0.761	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0.000
L. Biwa	1.000	0.000	0.000	0.526	0.474	0.879	0.121	0.000	0.000	0.000	1.000	0.000	0.019	0.815	0.167	0.000	1.000	0.000	0.976	0.024
Kumano R.	1.000	0.000	0.000	1.000	0.000	0.769	0.231	0.000	0.000	0.000	1.000	0.000	0.446	0.554	0.000	0.000	1.000	0.000	1.000	0.000
None R.	1.000	0.000	0.090	0.910	0.000	0.875	0.125	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0.000
Monobe R.																				
upper reach	1.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0.000
lower reach	1.000	0.000	0.000	1.000	0.000	0.988	0.012	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0.000
Kure R.	1.000	0.000	0.000	1.000	0.000	0.914	0.086	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0.000
Shimanto R.																				
upper reach	1.000	0.000	0.000	0.838	0.163	0.563	0.438	0.000	0.000	0.000	0.988	0.013	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0.000
middle reach	1.000	0.000	0.000	1.000	0.000	0.650	0.350	0.000	0.000	0.000	1.000	0.000	0.000	0.975	0.025	0.000	1.000	0.000	1.000	0.000
lower reach	1.000	0.000	0.000	1.000	0.000	0.788	0.213	0.000	0.000	0.000	0.988	0.013	0.000	0.988	0.013	0.000	1.000	0.000	1.000	0.000
Hitotsuse R.	1.000	0.000	0.023	0.965	0.012	0.718	0.282	0.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	1.000	0.000

TABLE 3. (Continued)

Location	*Ldh-1*		*Ldh-2*			*Ldh-3*			*S-Mdh-2*			*s-Me*		*6-Pgd*		*Pgm*			Sample size
	87	*100*	*−11*	*100*	*220*	*−188*	*−100*	*113*	*81*	*100*	*119*	*23*	*100*	*100*	*127*	*100*	*119*	*144*	
Abira R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.907	0.093	1.000	0.000	0.000	43
Otsuchi																			
upper reach	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	1.000	0.000	1.000	0.000	0.000	40
Otsuchi Bay	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.013	0.988	0.000	0.000	1.000	1.000	0.000	1.000	0.000	0.000	40
Hirose R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.012	0.988	0.000	0.000	1.000	1.000	0.000	1.000	0.000	0.000	41
Kinu R.	0.000	1.000	0.010	0.990	0.000	0.031	0.969	0.000	0.061	0.939	0.000	0.030	0.970	0.989	0.011	1.000	0.000	0.000	33
Kano R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.813	0.188	0.000	1.000	0.750	0.250	1.000	0.000	0.000	50
Aga R.	0.000	1.000	0.000	1.000	0.000	0.013	0.984	0.000	0.000	1.000	0.000	0.000	1.000	0.917	0.083	1.000	0.000	0.000	40
Iburibashi R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	1.000	0.000	1.000	0.000	0.000	40
Yoshii R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	1.000	0.000	1.000	0.000	0.000	41
Sufu R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.952	0.048	1.000	0.000	0.000	43
Chikugo R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.989	0.011	1.000	0.000	0.000	44
L. Biwa	0.034	0.966	0.000	1.000	0.000	0.000	0.955	0.045	0.000	0.739	0.261	0.000	1.000	0.236	0.764	1.000	0.000	0.000	44
Kumano R.	0.000	1.000	0.000	0.947	0.053	0.000	1.000	0.000	0.000	0.255	0.745	0.000	1.000	0.167	0.833	1.000	0.000	0.000	47
None R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.875	0.125	0.000	1.000	1.000	0.000	1.000	0.000	0.000	40
Monobe R.																			
upper reach	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.619	0.381	0.000	1.000	1.000	0.000	1.000	0.000	0.000	42
lower reach	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.683	0.317	0.000	1.000	0.988	0.012	1.000	0.000	0.000	41
Kure R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.413	0.588	0.000	1.000	1.000	0.000	1.000	0.000	0.000	40
Shimanto R.																			
upper reach	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.711	0.229	0.975	0.013	0.013	40
middle reach	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.700	0.300	0.975	0.025	0.000	40
lower reach	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.675	0.325	1.000	0.000	0.000	40
Hitotsuse R.	0.000	1.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000	0.991	0.009	0.000	1.000	0.808	0.192	1.000	0.000	0.000	54

TABLE 4. Estimates of Nei's genetic distance between pairs of 17 populations of *T. hakonensis* based on allelic frequencies at 20 loci

		17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2
1	Abira R.	.053	.064	.092	.086	.063	.135	.095	.004	.002	.005	.022	.007	.005	.009	.008	.005
2	Otsuchi Bay	.081	.094	.120	.113	.087	.172	.123	.013	.003	.003	.014	.002	.014	.006	.003	—
3	Hirose R.	.082	.095	.121	.114	.089	.169	.123	.016	.007	.009	.011	.003	.014	.002	—	
4	Kinu R.	.069	.080	.104	.097	.074	.152	.110	.014	.009	.011	.015	.008	.011	—		
5	Kano R.	.050	.058	.080	.079	.061	.103	.079	.010	.010	.016	.032	.016	—			
6	Aga R.	.083	.094	.123	.116	.090	.163	.115	.016	.005	.007	.005	—				
7	Iburibashi R.	.080	.090	.113	.104	.081	.158	.108	.025	.018	.016	—					
8	Yoshii R.	.062	.073	.095	.087	.065	.152	.102	.007	.001	—						
9	Sufu R.	.059	.070	.095	.088	.065	.146	.100	.005	—							
10	Chikugo R.	.031	.040	.062	.054	.035	.116	.076	—								
11	L. Biwa	.035	.028	.052	.045	.045	.035	—									
12	Kumano R.	.063	.055	.051	.060	.071	—										
13	None R.	.004	.007	.012	.003	—											
14	Monobe R.	.010	.012	.004	—												
15	Kure R.	.022	.025	—													
16	Shimanto R.	.001	—														
17	Hitotsuse R.	—															

the lower reach of each river were used for the comparison among populations. G_{ST} value which indicates the extent of differentiation of allelic frequencies over 17 populations was estimated to be 0.544.

D values which indicate the extent of differentiation between pairs of populations were shown in Table 5. D values between pairs of 17 populations ranged from 0.001 to 0.172. A dendrogram based on D values was further constructed to examine genetic relationships among the populations (Fig. 3). Three major clustering groups were discriminated on this dendrogram. Namely, the populations from the Abira River to the Kano River in the Pacific coast and from the Aga River to the Chikugo River in the coast of the Sea of Japan (including the Yoshii River in the side of the Inland Sea), those from the None River to the Hitotsuse River in the Pacific coast, and those of the Lake Biwa and the Kumano River formed each of the three clustering groups. We gave names to these local groups; 1) Northern Group, 2) Southern Group, and 3) Lake Biwa Group, respectively. From Table 5, D values between the Northern Group and the Southern Group ranged from 0.031 to 0.123 (0.081 on average). Similarly, the values between the Northern Group and the Lake Biwa Group, and between the Southern Group and the Lake Biwa Group ranged from 0.076 to 0.172 (0.125 on average), and from 0.028 to 0.070 (0.051 on average), respectively. On the other hand, D

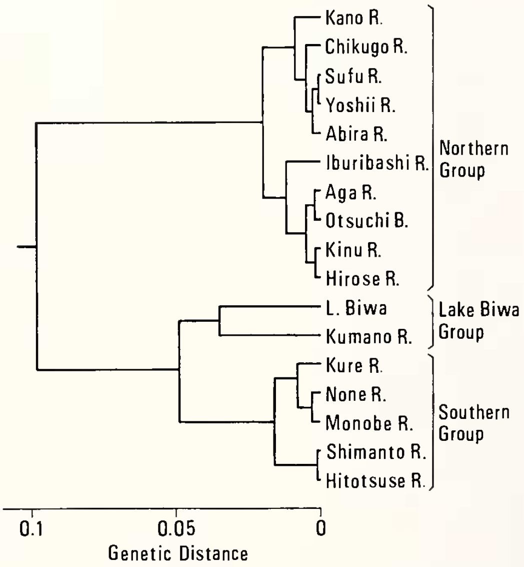

FIG. 3. A dendrogram based on Nei's genetic distance between pairs of 17 populations of *T. hakonensis*.

values between pairs of the populations within Northern, Southern, and Lake Biwa Groups ranged from 0.001 to 0.032 (0.010 on average), from 0.001 to 0.025 (0.010 on average), and 0.035, respectively. Thus, genetic distances between the local groups were considerably large compared with those within the groups.

DISCUSSION

Level of intrapopulational variability

Intrapopulational variability at protein level in various species of animals has been examined and that of fishes is close to the standard levels in vertebrates [18]. In the populations of *T. hakonensis,* proportion of loci polymorphic and heterozygosity were estimated to be 0.22±0.10 and 0.041±0.019, respectively (Table 3). These values are close to the mean values of Ostariophysi (0.16 and 0.045) and are within the range of fishes [18]. Therefore, it is regarded that intrapopulational variability of *T. hakonensis* is close to the standard levels in vertebrates. At the same time, this suggests that most of the populations in *T. hakonensis* has been maintained without extreme reduction of their size.

Level of interpopulational differentiation

Allelic frequencies were remarkably different among populations of three local groups in *T. hakonensis* (Table 3) and the extent of their differentiation was estimated by two kinds of indices. These data were further compared with those obtained in various animals. G_{ST} value among the populations of *T. hakonensis* was estimated as 0.544. Nei [16] reported G_{ST} value among populations in wild mice as 0.119, and that among populations in horseshoe crabs as 0.072, respectively. Thus, level of G_{ST} value in *T. hakonensis* is several times as high as the levels hitherto reported. This reveals that most of genetic variation exists within populations in the animals hitherto examined, whereas a half of genetic variation exists among populations in *T. hakonensis.*

Mean genetic distance between populations of different local groups in *T. hakonensis* was estimated as 0.051–0.125. Levels of genetic distance between local races or populations, and between subspecies of various vertebrates have been examined hitherto. Nei [16] reported D values between local races and between subspecies in wild mice as 0.010–0.024 and 0.194, respectively. In fish, D values between populations were reported as 0.001–0.006 for salmonid fish [19] and as 0.0007–0.0040 for red sea bream [20], and those between subspecies were reported as 0.11–0.19 for cyprinid fish, the genus *Campostoma* [21]. Thus, level of D values between populations of local groups in *T. hakonensis* is much higher than the levels between local races or populations hitherto reported, and is close to those between subspecies. These results are consistent with those of mitochondrial DNA, since mitochondrial genomes had been also highly differentiated among three populations of each local group [22]. Level of D values between populations within each local group of *T. hakonensis* is a little higher than or close to the levels hitherto reported.

In the other species of *Tribolodon, T. brandti* and *T. ezoe,* Sakai and Hamada [23] who examined populations from the waters of Hokkaido pointed out that allelic frequencies at a few loci were largely different from populations of Fukushima Pref. we had reported previously [7]. Hanzawa [24] estimated the genetic distance between these populations of two species based on allelic frequencies at 20 loci as 0.104 and 0.113, respectively. Thus, the high degree of interpopulational differentiation is also documented in the other two species of *Tribolodon.*

Geographical isolation of local groups

The extent of genetic differentiation among the local groups of *T. hakonensis* was remarkably high. Fishes of each local group of *T. hakonensis* are very similar in morphology. Nakamura [25] who examined morphological characters of *T. hakonensis* could not clearly show the regional differentiation, although he found slight differences among specimens collected from different regions in Japan. The present study on allozyme markers clearly showed the high degree of regional differentiation in the populations of *T. hakonensis.*

We considered two posssibilities which could explain the formation of these local groups. First is that any of populations of the local groups have undergone introgression and have accumulated genes derived from other related species. But, this is unlikely, because the local groups of *T. hakonensis* have been differentiated enough from the other species of *Tribolodon* at most of loci. Allelic differentiation between each local group of *T. hakonensis* and the other species of *Tribolodon* was observed at *m-Aat, s-Idh-1, 2, Ldh-1, 3, Pgm, Sp-2, 3* [6, 7, 24]. On the other hand, allelic differentiation among the local groups of *T. hakonensis* independently occurred at the other 5 loci, *Adh, α-Gdh-2, Gpi-2, s-Mdh-2, 6-Pgd*. Therefore, we cannot interpret that the other related species had taken part in the interpopulational differentiation of *T. hakonensis*.

The second possibility, which is the most probable, is that the populations of different local groups have been geographically isolated from each other since the time of the separation from their ancestral populations, and little gene flow has occurred among the populations of the groups. The dispersal of freshwater fishes is restricted by mountains, sea, etc., and regional differentiation occurs in their populations. Sakaizumi *et al.* [26] surveyed allozymic variation in Japanese wild populations of *Oryzias latipes*, and suggested that this case could be applied to interpopulational differentiation of this species because the boundary of the regions agreed well with watersheds or coastlines.

Distribution of the three local groups of *T. hakonensis* was shown in Figure 4. Their distribution shows distinct region specificity, and outline of their boundary agrees with watersheds or coast-

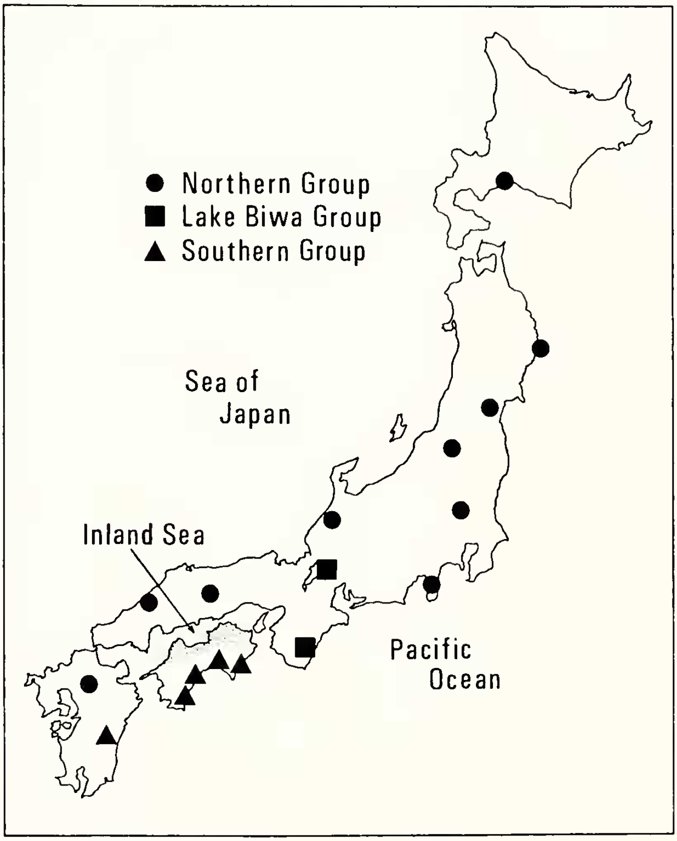

FIG. 4. Distribution of three local groups of *T. hakonensis*. *T. hakonensis* has not been originally distributed in the area designated by ▨.

lines. In the Southern Group of *T. hakonensis*, northeastern part of the boundary agrees with the Shikoku mountains, because *T. hakonensis* has not been originally distributed in the side of the Inland Sea of Shikoku ([27], Mizuno, N., personal communication). The Sikoku mountains may have restricted the dispersal of the Southern Group from the side of the Pacific Ocean to that of the Inland Sea. The restriction of dispersal by the Shikoku mountains is also suggested from the fact that faunae of freshwater fishes are largely different between the side of the Inland Sea and that of the Pacific Ocean in Shikoku [27, 28]. Considering that the Yoshii River population in the side of the Inland Sea of Honshu belongs to the Northern Group, the boundary of the Northern Group may agree with the coastline of the Inland Sea. In this regard, we speculate that *T. hakonensis* of the Northern Group has extended their distribution from the side of the Sea of Japan to that of the Inland Sea through plains around the Chugoku mountains. The reasons why the Northern Group had not extended their distribution to Shikoku through the Inland Sea is now obscure. But, we speculate one of the reasons is that *T. hakonensis* hardly migrates out of bays or coastal regions, as mentioned in detail later. Further investigation in the side of the Inland Sea of Honshu and Shikoku will give any suggestion.

Lake Biwa group included two populations, but gene constitution of these populations was somewhat different (Table 3, Fig. 3). Lake Biwa population which showed the unique gene constitution (Table 3) also suggests the geographical isolation. Lake Biwa had been originated in the Pliocene epoch, and has been maintained as the independent waters surrounded by watersheds [29]. Many endemic species, subspecies and local races are distributed in this lake, and it is considered that they have been isolated within this lake and have been differentiated here. It is likely that *T. hakonensis* has been also isolated within the lake for a considerably long period of time and has been remarkably differentiated from the other populations.

As stated above, the restriction of dispersal by watersheds may have affected the formation of the local groups. However, this cannot explain the reason why little gene flow is observed among the local groups although *T. hakonensis* can migrate through the sea regions. In this regard, we consider the following reasons. Though this species can migrate to the sea regions, it spawns in the middle reaches of rivers. This species is firmly dependent on the fresh waters, and may stay within bays or coastal regions without migrating to the open sea. Therefore, gene flow hardly occurs among populations of different local groups through the sea regions. This speculation is consistent with the observation that fish of this genus was distributed within the area of five meters in depth in Tokyo Bay [30]. Furthermore, this speculation may be supported by the observation that gene constitution of the amphidromous type of *T. hakonensis* was very similar to the landlocked type in the same waters (see Results). This observation suggests that the amphidromous type might have returned to its spawning ground in the middle reaches of the river without migrating to the other waters and had been mated with the landlocked type before these types were isolated by dums.

It is likely that the populations of different local groups have been geographically isolated from each other for a long period of time. Divergence time between three local groups based on Nei's genetic distance was estimated as approximately 200,000–600,000 years, and agreed with the divergence time based on the net nucleotide differences between mitochondrial DNA of three populations of each local group [22]. This time corresponds to the middle of diluvial epoch when the Japanese Islands had been strongly affected by ups and downs of the sea level with vicissitudes of glaciers and had connected with each other and the Asiatic Continent. It is likely that *T. hakonensis* had extended their distribution and that the local groups had been formed during this epoch. As for the dispersal and the differentiation of the local groups, we speculate as follows. Considering a fact that all of other related species of *Tribolodon* are distributed in the northern Japan and north of Japan at present, the ancestral population of *T. hakonensis* had been possibly distributed in or near these areas. First, the population of the Southern Group had extended their distribution to the southern Japan and had begun to be genetically differentiated from the ancestral population. Secondly, a part of the population of the Southern Group had been isolated and the Lake Biwa group had been differentiated. On the other hand, the Northern Group had arisen from the ancestral population which had stayed in the north, and had extented its distribution rapidly. Further study as for specimens sampled at other waters will make sure this hypothesis.

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